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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
C12N 15/82, 15/53, 9/02, A01H 5/00

A1

(11) International Publication Number: **WO 00/36126**

(43) International Publication Date: 22 June 2000 (22.06.00)

(21) International Application Number: PCT/EP99/09986

(22) International Filing Date: 15 December 1999 (15.12.99)

(30) Priority Data:
09/213,208 17 December 1998 (17.12.98) US

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: THIOREDOXIN AND GRAIN PROCESSING

(57) Abstract

The invention provides methods of processing grain, particularly corn and soybeans, utilizing thioredoxin and/or thioredoxin reductase to enhance extractability and recovery of starch and protein. The invention further provides transgenic plants expressing thermostable thioredoxin and/or thioredoxin reductase.

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THIOREDOXIN AND GRAIN PROCESSING

This invention relates to novel methods for grain processing to enhance protein and starch recovery, particularly in corn wet milling and soybean processing, as well as novel transgenic plants useful in such processes.

Thioredoxin (TRX) and thioredoxin reductase (TR) are enzymes that use NADPH to reduce disulfide bonds in proteins. Protein disulfide bonds play an important role in grain processing efficiencies, and in the quality of the products recovered from grain processing. Development of effective ways to eliminate or decrease the extent of protein disulfide bonding in grain would increase processing efficiencies. Additionally grain performance in livestock feed is also affected by inter- and intramolecular disulfide bonding: grain digestibility, nutrient availability and the neutralization of anti-nutritive factors (e.g., protease, amylase inhibitors etc.) would be increased by reducing the extent of disulfide bonding.

Expression of transgenic thioredoxin and/or thioredoxin reductase in corn and soybeans and the use of thioredoxin in grain processing, e.g., wet milling, is novel and provides an alternative method for reducing the disulfide bonds in seed proteins during industrial processing. The invention therefore provides grains with altered storage protein quality as well as grains that perform qualitatively differently from normal grain during industrial processing or animal digestion (both referred to subsequently as "processing").

This method of delivery of thioredoxin and/or thioredoxin reductase eliminates the need to develop exogenous sources of thioredoxin and/or thioredoxin reductase for addition during processing. A second advantage to supplying thioredoxin and/or thioredoxin reductase via the grains is that physical disruption of seed integrity is not necessary to bring the enzyme in contact with the storage or matrix proteins of the seed prior to processing or as an extra processing step.

Three modes of thioredoxin utilization in grain processing are provided:

1) Expression and action during seed development to alter the composition and quality of harvested grain;
2) Expression (but no activity) during seed development to alter the quality of the products upon processing;
3) Production of thioredoxin and/or thioredoxin reductase in grain that is used to alter the quality of other grain products by addition during processing.

The present invention thus provides:

— a method to increase efficiency of separation of starch and protein in a grain milling process, comprising steeping the grain at an elevated temperature in the presence of supplemental thioredoxin and/or thioredoxin reductase and separating the starch and protein components of the grain

— a method as mentioned hereinbefore wherein the grain includes grain from a transgenic plant wherein the transgene expresses thioredoxin and/or thioredoxin reductase, particularly a thermostable thioredoxin and/or thioredoxin reductase.

— a method as mentioned hereinbefore wherein the plant is selected from dicotyledonous or monocotyledonous plants, particularly from cereals and even more particularly from corn (*Zea mays*) and soybean.

The invention further provides transgenic plants.

In particular, the invention provides:

— a plant comprising a heterologous DNA sequence coding for a thioredoxin and/or thioredoxin reductase stably integrated into its nuclear or plastid DNA

— a plant as mentioned before wherein the thioredoxin and/or thioredoxin reductase is thermostable

— a plant as mentioned before wherein the thioredoxin and/or thioredoxin reductase is selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, and SEQ ID NO: 7

— a plant as mentioned herein before wherein the plant is selected from corn and soybean

The invention also provides:

— a plant expressible expression cassette comprising a coding region for a thioredoxin and/or thioredoxin reductase operably linked to promoter and terminator sequences which function in a plant

— a plant expressible expression cassette as mentioned before wherein the thioredoxin and/or thioredoxin reductase is thermostable

— a plant expressible expression cassette wherein the thioredoxin and/or thioredoxin reductase is selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, and SEQ ID NO: 7

The invention further provides

— method of producing grain comprising high levels of thioredoxin and/or thioredoxin reductase comprising transforming plants with an expression cassette as mentioned before

—a method of producing grain comprising high levels of thioredoxin and/or thioredoxin reductase comprising

pollinating a first plant comprising a heterologous expression cassette comprising a transactivator-regulated promoter regulated and operably linked to a DNA sequence coding for a thioredoxin and/or thioredoxin reductase, with pollen from a second plant comprising a heterologous expression cassette comprising a promoter operably linked to a DNA sequence coding for a transactivator capable of regulating said transactivator-regulated promoter; and recovering grain from the plant thus pollinated

In addition the invention provides:

— the use of plants or plant material according to the invention as animal feed.

The invention described herein is applicable to all grain crops, in particular corn, soybean, wheat, and barley, most particularly corn and soybean, especially corn. Expression of transgenic thioredoxin and/or thioredoxin reductase in grain is a means of altering the quality of the material (seeds) going into grain processing, altering the quality of the material derived from grain processing, maximizing yields of specific seed components during processing (increasing efficiency), changing processing methods, and creating new uses for seed-derived fractions or components from milling streams.

Wet-Milling

Wet milling is a process of separating the starch, protein and oil components of grain, most often cereals, for example corn. It is distinguished herein from dry milling, which is simply pulverizing grain. The first step in wet milling is usually steeping, wherein the grain is soaked in water under carefully controlled conditions to soften the kernels and facilitate separation of the components. The oil-bearing embryos float to the surface of the aqueous solution and are removed, and by a process of watering and dewatering, milling, screening, centrifuging and washing, the starch is separated from the protein and purified. The key difficulty is to loosen starch granules from the complicated matrix of proteins and cell wall material that makes up the endosperm of the grain. One reason for this difficulty is believed to be the presence of inter- or intramolecular disulfide bonds, which render the protein matrix less soluble and less susceptible to proteolytic enzymes and inhibit release of the

starch granules from the protein matrix in the grain. At present, the primary means for reducing these bonds is to steep the grain in the presence of sulfur dioxide, but this is costly, environmentally unfriendly, and not optimally effective.

Certain mutations exert beneficial effects on the protein matrix of corn kernel endosperm (*floury* and *opaque*), but impair kernel integrity. Transgenic thioredoxin expression provides some of these advantages without creating some of the kernel integrity problems associated with these mutations.

Post-harvest or processing-dependent activity of thioredoxin have equally beneficial effects. For example, in one embodiment, thioredoxin enzymes are targeted to and accumulated in cell compartments. Protein reduction occurs following physical disruption of the seed. In another embodiment, quiescent endosperm thioredoxin is activated upon steeping. In a preferred embodiment, the invention provides a plant expressing a transgenic thermostable thioredoxin and thioredoxin reductase, e.g. a thioredoxin and thioredoxin reductase derived from a hyperthermophilic organism, such that the thioredoxin and thioredoxin reductase are not significantly active except at high temperatures (e.g. greater than 50°C). In one embodiment, the thermostable thioredoxin and thioredoxin reductase are synergistic with saccharification via expression of other thermostable enzymes in endosperm.

Feed applications

Expression of transgenic thioredoxin and/or thioredoxin reductase in grain is also useful to improve grain characteristics associated with digestibility, particularly in animal feeds. Susceptibility of feed proteins to proteases is a function of time and of protein conformation. Kernel cracking is often used in feed formulation as is steam flaking. Both of these processes are designed to aid kernel digestibility. Softer kernels whose integrity can be disrupted more easily in animal stomachs are desirable. Conformational constraints and crosslinks between proteins are major determinants of protease susceptibility. Modifying these bonds by increased thioredoxin expression thereby aids digestion.

Corn dry milling/ masa

Protein content and quality are important determinants in flaking grit production and in masa production. Reduction of disulfide bonds alters the nature of corn flour such that it is suitable for use as a wheat substitute, especially flours made from high-protein white corn varieties.

Soybean crushing

Over half of the US soybean crop is crushed or milled, and the protein quality in the resulting low-fat soy flour or de-fatted soy flour (or grits) is important for subsequent processing. Protein yield and quality from soybean processing streams are economically important, and are largely dependent upon protein conformation. Increasing thioredoxin activity through expression of transgenic thioredoxin and/or thioredoxin reductase increases protein solubility, and thus increases yield, in the water-soluble protein fractions. Recovery is facilitated by aqueous extraction of de-fatted soybean meal under basic conditions. Enhancing thioredoxin activity through expression of transgenic thioredoxin and/or thioredoxin reductase also reduces the required pH for efficient extraction and thereby reduces calcium or sodium hydroxide inputs, as well as lowering the acid input for subsequent acid precipitation, allowing efficient recovery of proteins without alkali damage, and reducing water consumption and processing plant waste effluents (that contain substantial biological oxygen demand loads).

Protein redox status affects important functional properties supplied by soy proteins, such as solubility, water absorption, viscosity, cohesion/adhesion, gelation and elasticity. Fiber removal during soy protein concentrate production and soy protein isolate hydrolysis by proteases is enhanced by increasing thioredoxin activity as described herein. Similarly, as described for corn above, increasing thioredoxin activity through expression of transgenic thioredoxin and/or thioredoxin reductase enhances the functionality of enzyme-active soy flours and the digestibility of the soybean meal fraction and steam flaking fraction in animal feeds.

Modification of protein quality during seed development and during processing both be provided, although it is preferred that the transgenic thioredoxin and/or thioredoxin reductase be targeted to a cell compartment and be thermostable, as described above, to avoid significant adverse effects on storage protein accumulation will be encountered as a result of thioredoxin activity during seed development. Alternately, the thioredoxin may be added as a processing enzyme, as (in contrast to corn wet milling) breaking the disulfide bonds is not necessary until after grain integrity is destroyed (crushing and oil extraction).

Selection of thioredoxin and thioredoxin reductase for heterologous expression:

Thioredoxin, thioredoxin reductase and protein disulfide isomerase (PDI) genes are found in eukaryotes, eubacteria as well as archea, including hyperthermophilic organisms such as *Methanococcus jannaschii* and *Archaeoglobus fulgidus*. Selection of a particular gene

depends in part on the desired application. For the methods of the present invention, preferred thioredoxins have the following characteristics:

1. Heat stability

Thioredoxin and related proteins from hyperthermophiles are found to have increased stability at high temperatures (>50°C) and relatively low activity at ambient temperatures. Expression of TRX and/or TR from hyperthermophiles, for example from archea such as *Methanococcus jannaschii* and *Archaeoglobus fulgidus* or other hyperthermophiles is preferred for expression during seed development, so that the thioredoxin activity is not markedly increased until the grain is steeped or processed at elevated temperature. Most grain processing methods involve, or are compatible with, a high temperature step. Thermostable thioredoxin and thioredoxin reductase is therefore preferred. By thermostable is meant that the enzyme is preferentially active at high temperatures, e.g., temperatures greater than 40°C, most preferably greater than 50°C, e.g. 45-60°C for wet milling, or even higher, e.g., 45-95°C.

2. Substrate specificity

It is also possible to reduce undesirable effects on seed development by selection of a thioredoxin that acts preferentially on certain proteins such as the structural protein in the matrix and has low activity with essential metabolic enzymes. Various TRX's have been shown to differ in reactivity with enzymes that are under redox control. Thus it is possible to select a TRX that will primarily act on the desired targets, minimizing undesirable side-effects of over expression.

Suitable thermostable thioredoxins and thioredoxin reductases include the following:

Sequence of thioredoxin from *Methanococcus jannaschii*, (SEQ ID NO:1; gi|1591029)

MSKVKIELFTSPMCPHCPAAKRVVEEVANEMPDAVEVEYINVMENPQKAMEYGIMAVPTIVI
NGDVEFIGAPTKEALVEAIKKRL

Sequence of thioredoxin from *Archaeoglobus fulgidus* (SEQ ID NO:2; gi|2649903)(trx-1)

MPMVRKAAFYAIAVISGVLAAVVGNALYHNFNSDLGAQAKIYFFYSDSCPHCREVKPYVEEF
AKTHNLTWCNVAEMDANCSKIAQEFGIKYVPTLVIMDEEAHVFVGSDEVRTAIEGMK

Sequence of thioredoxin from *Archaeoglobus fulgidus* (SEQ ID NO:3; gi|2649838) (trx-2)

MVFTSKYCPYCRAFEKVVERLMGELNGTVEFEVVDVDEKRELAEKYEVLMLPTLVLADGDE
VLGGFMGFADYKTAREAILEQISAFLKPDYKN

Sequence of thioredoxin from *Archaeoglobus fulgidus* (SEQ ID NO:4; gi|2649295) (trx-3)
MDELELIRQKKLKEMMQKMSGEEKARKVLDSPVKLNSSNFDETLKNNENVVVDFWAEWC
MPCKMIAPVIEELAKEYAGKVVFGKLNTDENPTIAARYGISAIPTLIFFKKGKPVDQLVGAMPK
SELKRWVQRNL

Sequence of thioredoxin from *Archaeoglobus fulgidus* (SEQ ID NO:5; gi|2648389) (trx-4)
MERLNSERFREVIQSDKLVVVDFYADWCMPCRYISPILEKLSKEYNGEVEFYKLNVDENQD
VAFEYGIASIPTVLFFRNGKVVGGFIGAMPESAVRAEIEKALGA

Sequence of thioredoxin reductase (trxB) from *Methanococcus jannaschii* (SEQ ID NO:6: gi|1592167)
MIHDTIIIGAGPGGLTAGIYAMRGKLNALCIEKENAGGRIAEAGIVENYPGFEEIRGYELAEKF
KNHAEKFKLPIIYDEVIKIETKERPFKVITKNSEYLTKTIVIATGTKPKKLGLNEDKFIGRGISYC
TMCDAFFYLNKEVIVIGRDTPAIMSAINLKDIAKKVIVITDKSELKAAESIMLDKLKEANNVEIIY
NAKPLEIVGEERAEGVKISVNGKEEIIKADGIFISLGHVPNTEFLKDSGIELDKKGFIKTDENCR
TNIDGIYAVGDVRGGVMQVAKAVGDGCVAMANIIKYLQKL

Sequence of thioredoxin reductase from *Archaeoglobus fulgidus* (SEQ ID NO:7; gi|2649006) (trxB)
MYDVAIIGGGPAGLTAALYSARYGLKTVFFETVDPVSQLSLAAKIENYPGFEGSGMELLEKM
KEQAVKAGAEWKLEKVERVERNGETFTVIAEGGEYEAKAIIVATGGKHKEAGIEGESAFIGR
GVSYCATCDGNFFRGKKVIVYGSGKEAIEDAIYLHDIGCEVTIVSRTPSFRAEKALVEEVEKR
GIPVHYSTTIRKIIGSGKVEKVVAYNREKKEEFEIEADGIFVAIGMRPATDVVAELGVERDSM
GYIKVDKEQRTNVEGVFAAGDCCDNPLKQVVTACGDGAVAAYSAYKYLTS

The genes which encode these proteins for use in the present invention are preferably designed by back-translation using plant preferred codons, to enhance G-C content and remove detrimental sequences, as more fully described below. The activity of the proteins may be enhanced by DNA shuffling or other means, as described below. The invention therefore comprises proteins derived from these proteins, especially proteins which are substantially similar which retain thioredoxin or thioredoxin reductase activity.

For engineering thioredoxin expression in seeds for activity during grain development, promoters which direct seed-specific expression of TRX and TR are preferred, as is targeting to the storage so that the enzyme will have the desired effects on storage proteins, which may be desirable in some applications. In the present invention, however, it is more generally desirable to engineer thioredoxin and/or thioredoxin reductase expression in seeds for accumulation and inactivity during grain development. Several strategies are employed to create seeds that express transgenic thioredoxin and/or thioredoxin reductase without having a significant impact on normal seed development, e.g.:

(i) To compartmentalize active thioredoxin or thioredoxin reductase such that it does not significantly interact with the target proteins, for example by targeting to or expression in amyloplasts. Plastid targeting sequences are used to direct accumulation in the amyloplast. Alternatively, the thioredoxin and/or thioredoxin reductase is targeted to an extracellular location in cell walls using secretion signals. Or finally, in the case of monocots, expression in cell types such as aleurone during seed development is used to keep the thioredoxin and/or thioredoxin reductase away from the storage components of the rest of the endosperm.

(ii) To engineer the expression of thioredoxin and/or thioredoxin reductase from thermophilic organisms. Enzymes which have little or no activity at ambient temperatures (as high as 38-39°C in the field) are less likely to cause problems during development. Preferably, therefore, the enzymes are active primarily at high temperatures, e.g., temperatures greater than 40°C, most preferably 45-60°C for wet milling, or even higher, e.g., 45-95°C.

(iii) To place the thioredoxin and/or thioredoxin reductase under control of an inducible promoter, for example a chemically-inducible promoter, a wound-inducible promoter, or a transactivator-regulated promoter which is activated upon pollination by a plant expressing the transactivator.

(iv) To utilize thioredoxin having specific requirements for a particular thioredoxin reductase, such that activity of the thioredoxin or thioredoxin reductase is suitably regulated via availability of the appropriate thioredoxin reductase or thioredoxin, respectively. For example, the thioredoxin and thioredoxin reductase are expressed in different plants, so that the active combination is only available in the seed upon pollination by the plant expressing the complimentary enzyme. Alternatively, the thioredoxin or thioredoxin reductase is sequestered in the cell, for example in a plastid, vacuole, or apoplast, as described above, so that it does not become available until the grain is processed.

Methods of grain processing

The invention thus provides a novel method of enhancing separation of the starch from the protein matrix, using thioredoxin and/or thioredoxin reductase. In a first embodiment, thioredoxin activity is found to be useful in a variety of seed processing applications, including wet milling, dry milling, oilseed processing, soybean processing, wheat processing and flour/dough quality, most especially the wet milling of grains, in particular corn.

Accordingly, the invention provides a method

- to improve milling efficiency or increase milling yield,
- to increase efficiency of separation of starch and protein,
- to enhance yields of starch and soluble proteins from grain, or
- to increase protein solubility in water or other solvents

comprising steeping grain in the presence of supplemental thioredoxin and/or thioredoxin and separating the starch and protein components of the grain.

Typically, steeping occurs before milling, but may occur afterwards, and there may be more than one milling or steeping step in the process method extraction and increase protein yield from seeds during the steep or points after steeping. Preferably, the supplemental thioredoxin and/or thioredoxin reductase is provided by expression of a transgene in the plant from which the grain is harvested.

The invention further provides:

- the use of thioredoxin or thioredoxin reductase in a method to improve milling efficiency or increase milling yield of starches or proteins, for example in any of the methods described above
- steepwater comprising an amount of thioredoxin and/or thioredoxin reductase effective to facilitate separation of starch from protein in grain
- grain which has been exposed to thioredoxin an amount effective to facilitate separation of starch from protein; and
- starch or protein which has been produced by the method described above.

The activity of the thioredoxin in the above method may be enhanced by supplementing the steepwater with thioredoxin reductase and/or NADPH. Other components normally present

in steepwater for wet milling may also be present, such as bacteria which produce lactic acid. Preferably, the steeping is carried out at a temperature of about 52°C for a period of 22-50 hours, so it is desirable that the thioredoxin is stable under these conditions.
The grain may be a dicotyledonous seed, for example, an oil seed, e.g., soybean, sunflower or canola, preferably soybean; or may be a monocotyledonous seed, for example a cereal seed, e.g., corn, wheat, oats, barley, rye or rice, most preferably corn.
The thioredoxin may be any protein bearing thiol groups which can be reversibly oxidized to form disulfide bonds and reduced by NAPDH in the presence of a thioredoxin reductase. Preferably the thioredoxin is derived from a thermophilic organism, as described above.
Thioredoxin and/or thioredoxin reductase for use in the instant invention is suitably produced in an engineered microbe, e.g. a yeast or Aspergillus, or in an engineered plant capable of very high expression, e.g. in barley, e.g., under control of a promoter active during malting, such as a high pI alpha-amylase promoter or other gibberellin-dependent promoters. The thioredoxin (in excreted or extracted form or in combination with the producer organism or parts thereof) is then added to the steepwater.
As an alternative or supplement to adding the thioredoxin to the steepwater, the enzyme can be expressed directly in the seed that is to be milled. Preferably, the enzyme is expressed during grain maturation or during a conditioning process.

Accordingly, in a further embodiment, the invention provides

- a method of making thioredoxin on an industrial scale in a transgenic organism, e.g., a plant, e.g., a cereal, such as barley or corn, or a microorganism, e.g., a yeast or Aspergillus, for example a method comprising the steps of cultivating a transgenic organism having a chimeric gene which expresses thioredoxin, and optionally isolating or extracting the thioredoxin
- a method of using transgenic plants that produce elevated quantities of thioredoxin during seed maturation or germination such that the quality of the proteins in that seed are affected by the endogenously synthesized thioredoxin during seed development, or during the steeping process, thereby eliminating or reducing the need for conditioning with exogenous chemicals or enzymes prior to milling
- a method of making transgenic plants that produce elevated quantities of thioredoxin during seed maturation or germination such that the quality of the proteins in that seed are affected by the thioredoxin during seed development or during the steeping process,

thereby eliminating or reducing the need for conditioning with exogenous chemicals or enzymes prior to milling

- a method for milling grain that uses transgenic seed containing thioredoxin, that results in higher starch and soluble protein yields.

Expression of thioredoxin and thioredoxin reductase in transgenic organisms

The invention further comprises a transgenic organism having in its genome a chimeric expression cassette comprising a coding region encoding a thermostable thioredoxin or thioredoxin reductase under operative control of a promoter.

Preferably, the transgenic organism is a plant which expresses a thioredoxin and/or thioredoxin reductase in a form not naturally occurring in plants of that species or which expresses thioredoxin at higher levels than naturally occur in a plant of that species. Preferably, the thioredoxin is expressed in the seed during seed development, and is therefore preferably under control of a seed specific promoter. Optionally, expression of the thioredoxin is placed under control of an inducible or transactivator-regulated promoter, so that expression is activated by chemical induction or hybridization with a transactivator when desired. The thioredoxin is suitably targeted to the vacuoles of the plant by fusion with a vacuole targeting sequence.

In the present invention, thioredoxin coding sequences are fused to promoters active in plants and transformed into the nuclear genome or the plastid genome. The promoter is preferably a seed specific promoter such as the gamma-zein promoter. The promoter may alternatively be a chemically-inducible promoter such as the tobacco PR-1a promoter; or may be a chemically induced transactivator-regulated promoter wherein the transactivator is under control of a chemically-induced promoter; however, in certain situations, constitutive promoters such as the CaMV 35S or Gelvin promoter may be used. With a chemically inducible promoter, expression of the thioredoxin genes transformed into plants may be activated at an appropriate time by foliar application of a chemical inducer.

Alternatively, the thioredoxin coding sequence is under control of a transactivator-regulated promoter, and expression is achieved by crossing the plant transformed with this sequence with a second plant expressing the transactivator. In a preferred form of this method, the first plant containing the thioredoxin coding sequence is the seed parent and is male sterile, while the second plant expressing the transactivator is the pollinator. Expression of thioredoxin in seeds is achieved by interplanting the first and second plants, e.g., such that the first plant is pollinated by the second and thioredoxin is expressed in the seeds of the

first plant by activation of the transactivator-regulated promoter with the transactivator expressed by the transactivator gene from the second parent.

The invention thus provides a plant which expresses a thioredoxin and/or thioredoxin reductase, e.g. a thioredoxin and/or thioredoxin reductase not naturally expressed in plants, for example a plant comprising a heterologous DNA sequence coding for a thioredoxin stably integrated into its nuclear or plastid DNA, preferably under control of an inducible promoter, e.g., a chemically-inducible promoter, for example either operably linked to the inducible promoter or under control of transactivator-regulated promoter wherein the corresponding transactivator is under control of the inducible promoter or is expressed in a second plant such that the promoter is activated by hybridization with the second plant; wherein the thioredoxin or thioredoxin reductase is preferably thermostable; such plant also including seed therefor, which seed is optionally treated (e.g., primed or coated) and/or packaged, e.g. placed in a bag with instructions for use, and seed harvested therefrom, e.g., for use in a milling process as described above.
The transgenic plant of the invention may optionally further comprise genes for enhanced production of thioredoxin reductase and/or NADPH.

The invention further provides:

- a method for producing thioredoxin comprising cultivating a thioredoxin-expressing plant as described above
- a method for producing starch and/or protein comprising extracting starch or protein from seed harvested from a plant as described above; and
- a method for wet milling comprising steeping seed from a thioredoxin-expressing plant as described above and extracting starch and/or protein therefrom.

The invention further provides:

- a plant expressible expression cassette comprising a coding region for a thioredoxin or thioredoxin reductase, preferably a thioredoxin derived from a thermophilic organism, e.g., from an archea, for example from *M. jannaschii* or *A. fulgidus*, e.g., as described above, wherein the coding region is preferably optimized to contain plant preferred codons, said coding region being operably linked to promoter and terminator sequences which function in a plant, wherein the promoter is preferably a seed specific promoter or an inducible promoter, e.g., a chemically inducible or transactivator-regulated promoter;

for example a plastid or nuclear expressible expression cassette comprising a promoter, e.g., a transactivator-regulated promoter regulated by a nuclear transactivator (e.g., the T7 promoter when the transactivator is T7 RNA polymerase the expression of which is optionally under control of an inducible promoter)

- a vector comprising such a plant expressible expression cassette
- a plant transformed with such a vector; or
- a transgenic plant which comprises in its genome, e.g., its nuclear or plastid genome, such a plant expressible expression cassette.

The invention also comprises a method of producing grain comprising high levels of thioredoxin or thioredoxin reductase comprising pollinating a first plant comprising a heterologous expression cassette comprising a transactivator-regulated promoter regulated and operably linked to a DNA sequence coding for a thioredoxin or thioredoxin reductase, the first plant preferably being emasculated or male sterile; with pollen from a second plant comprising a heterologous expression cassette comprising a promoter operably linked to a DNA sequence coding for a transactivator capable of regulating said transactivator-regulated promoter; recovering grain from the plant thus pollinated.

DEFINITIONS

In order to ensure a clear and consistent understanding of the specification and the claims, the following definitions are provided:

"Expression cassette" as used herein means a DNA sequence capable of directing expression of a gene in plant cells, comprising a promoter operably linked to a coding region of interest which is operably linked to a termination region. The coding region usually codes for a protein of interest but may also code for a functional RNA of interest, for example antisense RNA or a nontranslated RNA that, in the sense or antisense direction, inhibits expression of a particular gene, e.g., antisense RNA. The gene may be chimeric, meaning that at least one component of the gene is heterologous with respect to at least one other component of the gene. The gene may also be one which is naturally occurring but has been obtained in a recombinant form useful for genetic transformation of a plant. Typically, however, the expression cassette is heterologous with respect to the host, i.e., the particular DNA sequence of the expression cassette does not occur naturally in the host

cell and must have been introduced into the host cell or an ancestor of the host cell by a transformation event.

A "nuclear expression cassette" is an expression cassette which is integrated into the nuclear DNA of the host.

A "plastid expression cassette" is an expression cassette which is integrated into the plastid DNA of the host. A plastid expression cassette as described herein may optionally comprise a polycistronic operon containing two or more cistronic coding sequences of interest under control of a single promoter, e.g., a transactivator-regulated promoter, e.g., wherein one of the coding sequences of interest encodes an antisense mRNA which inhibits expression of clpP or other plastid protease, thereby enhancing accumulation of protein expressed the other coding sequence or sequences of interest.

"Heterologous" as used herein means "of different natural origin". For example, if a plant is transformed with a gene derived from another organism, particularly from another species, that gene is heterologous with respect to that plant and also with respect to descendants of the plant which carry that gene.

"Homoplastidic" refers to a plant, plant tissue or plant cell wherein all of the plastids are genetically identical. This is the normal state in a plant when the plastids have not been transformed, mutated, or otherwise genetically altered. In different tissues or stages of development, the plastids may take different forms, e.g., chloroplasts, proplastids, etioplasts, amyloplasts, chromoplasts, and so forth.

An "inducible promoter" is a promoter which initiates transcription only when the plant is exposed to some particular external stimulus, as distinguished from constitutive promoters or promoters specific to a specific tissue or organ or stage of development. Particularly preferred inducible promoters for the present invention are chemically-inducible or transactivator-regulated promoters. Chemically inducible promoters include plant-derived promoters, such as the promoters in the systemic acquired resistance pathway, for example the PR promoters, e.g., the PR-1, PR-2, PR-3, PR-4, and PR-5 promoters, especially the tobacco PR-1a promoter and the *Arabidopsis* PR-1 promoter, which initiate transcription when the plant is exposed to BTH and related chemicals. *See* US Patent 5,614,395, incorporated herein by reference, and WO 98/03536, incorporated herein by reference. Chemically-inducible promoters also include receptor-mediated systems, e.g., those derived from other organisms, such as steroid-dependent gene expression, copper-dependent gene expression, tetracycline-dependent gene expression, and particularly the expression system utilizing the USP receptor from *Drosophila* mediated by juvenile growth hormone and its

agonists, described in PCT/EP 96/04224, incorporated herein by reference, as well as systems utilizing combinations of receptors, e.g., as described in PCT/EP 96/00686, incorporated herein by reference. Chemically inducible promoters may be directly linked to the thioredoxin gene or the thioredoxin gene may be under control of a transactivator-regulated promoter while the gene for the transactivator is under control of a chemically inducible promoter. *See generally*, C. Gatz, "Chemical Control of Gene Expression", Annu. Rev. Plant Physiol. Plant Mol. Biol. (1997) 48: 89-108, the contents of which are incorporated herein by reference. Transactivator regulated promoters are described more fully below, and may also be induced by hybridization of a plant comprising the thioredoxin gene under control of a transactivator-regulated promoter with a second plant expressing the transactivator.

An "isolated DNA molecule" is a nucleotide sequence that, by the hand of man, exists apart from its native environment and is therefore not a product of nature. An isolated nucleotide sequence may exist in a purified form or may exist in a non-native environment such as, for example, a transgenic host cell.

A "protein" as defined herein is the entire protein encoded by the corresponding nucleotide sequence, or is a portion of the protein encoded by the corresponding portion of the nucleotide sequence.

An "isolated protein" is a protein that is encoded by an isolated nucleotide sequence and is therefore not a product of nature. An isolated protein may exist in a purified form or may exist in a non-native environment, such as a transgenic host cell, wherein the protein would not normally expressed or would be expressed in a different form or different amount in an isogenic non-transgenic host cell.

A "plant" refers to any plant or part of a plant at any stage of development, and is specifically intended to encompass plants and plant material which have been damaged, crushed or killed, as well as viable plants, cuttings, cell or tissue cultures, and seeds.

"DNA shuffling" is a method to introduce mutations or rearrangements, preferably randomly, in a DNA molecule or to generate exchanges of DNA sequences between two or more DNA molecules, preferably randomly. The DNA molecule resulting from DNA shuffling is a shuffled DNA molecule that is a non-naturally occurring DNA molecule derived from at least one template DNA molecule. The shuffled DNA encodes an enzyme modified with respect to the enzyme encoded by the template DNA, and preferably has an altered biological activity with respect to the enzyme encoded by the template DNA.

In its broadest sense, the term "substantially similar", when used herein with respect to a nucleotide sequence, means a nucleotide sequence corresponding to a reference nucleotide sequence, wherein the corresponding sequence encodes a polypeptide having substantially the same structure and function as the polypeptide encoded by the reference nucleotide sequence, e.g. where only changes in amino acids not affecting the polypeptide function occur. Desirably the substantially similar nucleotide sequence encodes the polypeptide encoded by the reference nucleotide sequence. The percentage of identity between the substantially similar nucleotide sequence and the reference nucleotide sequence desirably is at least 80%, more desirably at least 85%, preferably at least 90%, more preferably at least 95%, still more preferably at least 99%. Sequence comparisons are carried out using a Smith-Waterman sequence alignment algorithm (see e.g. Waterman, M.S. Introduction to Computational Biology: Maps, sequences and genomes. Chapman & Hall. London: 1995. ISBN 0-412-99391-0, or at http://www-hto.usc.edu/software/seqaln/index.html). The localS program, version 1.16, is used with following parameters: match: 1, mismatch penalty: 0.33, open-gap penalty: 2, extended-gap penalty: 2.

A nucleotide sequence "substantially similar" to reference nucleotide sequence hybridizes to the reference nucleotide sequence in 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50°C with washing in 2X SSC, 0.1% SDS at 50°C, more desirably in 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50°C with washing in 1X SSC, 0.1% SDS at 50°C, more desirably still in 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50°C with washing in 0.5X SSC, 0.1% SDS at 50°C, preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50°C with washing in 0.1X SSC, 0.1% SDS at 50°C, more preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50°C with washing in 0.1X SSC, 0.1% SDS at 65°C.

The term "substantially similar", when used herein with respect to a protein, means a protein corresponding to a reference protein, wherein the protein has substantially the same structure and function as the reference protein, e.g. where only changes in amino acids not affecting the polypeptide function occur. When used for a protein or an amino acid sequence the percentage of identity between the substantially similar and the reference protein or amino acid sequence desirably is at least 80%, more desirably 85%, preferably at least 90%, more preferably at least 95%, still more preferably at least 99%.

A "transactivator" is a protein which, by itself or in combination with one or more additional proteins, is capable of causing transcription of a coding region under control of a

corresponding transactivator-regulated promoter. Examples of transactivator systems include phage T7 gene 10 promoter, the transcriptional activation of which is dependent upon a specific RNA polymerase such as the phage T7 RNA polymerase. The transactivator is typically an RNA polymerase or DNA binding protein capable of interacting with a particular promoter to initiate transcription, either by activating the promoter directly or by inactivating a repressor gene, e.g., by suppressing expression or accumulation of a repressor protein. The DNA binding protein may be a chimeric protein comprising a binding region (e.g., the GAL4 binding region) linked to an appropriate transcriptional activator domain. Some transactivator systems may have multiple transactivators, for example promoters which require not only a polymerase but also a specific subunit (sigma factor) for promoter recognition, DNA binding, or transcriptional activation. The transactivator is preferably heterologous with respect to the plant.

Modification of Microbial Genes to Optimize Nuclear Expression in Plants

If desired, the cloned thioredoxin genes described in this application can be modified for expression in transgenic plant hosts. For example, the transgenic expression in plants of genes derived from microbial sources may require the modification of those genes to achieve and optimize their expression in plants. In particular, bacterial ORFs that encode separate enzymes but which are encoded by the same transcript in the native microbe are best expressed in plants on separate transcripts. To achieve this, each microbial ORF is isolated individually and cloned within a cassette which provides a plant promoter sequence at the 5' end of the ORF and a plant transcriptional terminator at the 3' end of the ORF. The isolated ORF sequence preferably includes the initiating ATG codon and the terminating STOP codon but may include additional sequence beyond the initiating ATG and the STOP codon. In addition, the ORF may be truncated, but still retain the required activity; for particularly long ORFs, truncated versions which retain activity may be preferable for expression in transgenic organisms.

By "plant promoter" and "plant transcriptional terminator" it is intended to mean promoters and transcriptional terminators which operate within plant cells. This includes promoters and transcription terminators which may be derived from non-plant sources such as viruses (examples include promoters and terminators derived from the Cauliflower Mosaic Virus or from opine synthase genes in Agrobacterium Ti or Ri plasmids).

In some cases, modification to the ORF coding sequences and adjacent sequence will not be required, in which case it is sufficient to isolate a fragment containing the ORF of interest

and to insert it downstream of a plant promoter. Preferably, however, adjacent microbial sequences left attached upstream of the ATG and downstream of the STOP codon should be minimized or eliminated. In practice, such construction may depend on the availability of restriction sites.

In other cases, the expression of genes derived from microbial sources may provide problems in expression. These problems have been well characterized in the art and are particularly common with genes derived from certain sources such as *Bacillus*. The modification of such genes can be undertaken using techniques now well known in the art. The following problems are typical of those that may be encountered:

1. Codon Usage

The preferred codon usage in plants differs from the preferred codon usage in certain microorganisms. Comparison of the usage of codons within a cloned microbial ORF to usage in plant genes (and in particular genes from the target plant) will enable an identification of the codons within the ORF which should preferably be changed. Typically plant evolution has tended towards a strong preference of the nucleotides C and G in the third base position of monocotyledons, whereas dicotyledons often use the nucleotides A or T at this position. By modifying a gene to incorporate preferred codon usage for a particular target transgenic species, many of the problems described below for GC/AT content and illegitimate splicing will be overcome.

2. GC/AT Content

Plant genes typically have a GC content of more than 35%. ORF sequences which are rich in A and T nucleotides can cause several problems in plants. Firstly, motifs of ATTTA are believed to cause destabilization of messages and are found at the 3' end of many short-lived mRNAs. Secondly, the occurrence of polyadenylation signals such as AATAAA at inappropriate positions within the message is believed to cause premature truncation of transcription. In addition, monocotyledons may recognize AT-rich sequences as splice sites (see below).

3. Sequences Adjacent to the Initiating Methionine

Plants differ from microorganisms in that their messages do not possess a defined ribosome binding site. Rather, it is believed that ribosomes attach to the 5' end of the message and scan for the first available ATG at which to start translation. Nevertheless, it is believed that there is a preference for certain nucleotides adjacent to the ATG and that expression of microbial genes can be enhanced by the inclusion of a eukaryotic consensus

translation initiator at the ATG. Clontech (1993/1994 catalog, page 210) have suggested the sequence GTCGACCATGGTC (SEQ ID NO: 8) as a consensus translation initiator for the expression of the *E. coli uidA* gene in plants. Further, Joshi (NAR 15: 6643-6653 (1987)) has compared many plant sequences adjacent to the ATG and suggests the consensus TAAACAATGGCT (SEQ ID NO: 9). In situations where difficulties are encountered in the expression of microbial ORFs in plants, inclusion of one of these sequences at the initiating ATG may improve translation. In such cases the last three nucleotides of the consensus may not be appropriate for inclusion in the modified sequence due to their modification of the second AA residue. Preferred sequences adjacent to the initiating methionine may differ between different plant species. A survey of 14 maize genes located in the GenBank database provided the following results:

Position Before the Initiating ATG in 14 Maize Genes:

	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1
C	3	8	4	6	2	5	6	0	10	7
T	3	0	3	4	3	2	1	1	1	0
A	2	3	1	4	3	2	3	7	2	3
G	6	3	6	0	6	5	4	6	1	5

This analysis can be done for the desired plant species into which the thioredoxin or thioredoxin reductase genes are being incorporated, and the sequence adjacent to the ATG modified to incorporate the preferred nucleotides.

4. Removal of Illegitimate Splice Sites

Genes cloned from non-plant sources and not optimized for expression in plants may also contain motifs which may be recognized in plants as 5' or 3' splice sites, and be cleaved, thus generating truncated or deleted messages. These sites can be removed using the techniques described in patent application WO 97/02352, hereby incorporated by reference.

Techniques for the modification of coding sequences and adjacent sequences are well known in the art. In cases where the initial expression of a microbial ORF is low and it is deemed appropriate to make alterations to the sequence as described above, then the

construction of synthetic genes can be accomplished according to methods well known in the art. These are, for example, described in the published patent disclosures EP 0 385 962, EP 0 359 472 and WO 93/07278. In most cases it is preferable to assay the expression of gene constructions using transient assay protocols (which are well known in the art) prior to their transfer to transgenic plants.

A major advantage of plastid transformation is that plastids are generally capable of expressing bacterial genes without substantial modification. Codon adaptation, etc. as described above is not required, and plastids are capable of expressing multiple open reading frames under control of a single promoter.

Construction of Plant Transformation Vectors and Selectable Markers

Numerous transformation vectors are available for plant transformation, and the genes of this invention can be used in conjunction with any such vectors. The selection of vector for use will depend upon the preferred transformation technique and the target species for transformation. For certain target species, different antibiotic or herbicide selection markers may be preferred. Selection markers used routinely in transformation include the *nptII* gene which confers resistance to kanamycin and related antibiotics (Vieira & Messing, Gene 19: 259-268 (1982); Bevan *et al.*, Nature 304:184-187 (1983)), the *bar* gene which confers resistance to the herbicide pho*Sphl*nothricin (White *et al.*, Nucl Acids Res 18: 1062 (1990), Spencer *et al.* Theor Appl Genet 79: 625-631(1990)), the *hpt* gene which confers resistance to the antibiotic hygromycin (Blochinger & Diggelmann, Mol Cell Biol 4: 2929-2931), the *dhfr* gene, which confers resistance to methotrexate (Bourouis *et al.*, EMBO J. 2(7): 1099-1104 (1983)); and the mannose-6-phosphate isomerase gene which confers the ability to metabolize mannose, as described in US 5767378.

Requirements for Construction of Plant Expression Cassettes

Gene sequences intended for expression in transgenic plants are firstly assembled in expression cassettes behind a suitable promoter and upstream of a suitable transcription terminator. These expression cassettes can then be easily transferred to the plant transformation vectors described above.

1. Promoter Selection

The selection of promoter used in expression cassettes will determine the spatial and temporal expression pattern of the transgene in the transgenic plant. Selected promoters will express transgenes in specific cell types (such as leaf epidermal cells, mesophyll cells,

root cortex cells) or in specific tissues or organs (roots, seeds, leaves or flowers, for example) and this selection will reflect the desired location of biosynthesis of the thioredoxin. In the present invention, seed specific promoters are preferred. Alternatively, the selected promoter may drive expression of the gene under a light-induced or other temporally regulated promoter. A further alternative is that the selected promoter be inducible by an external stimulus, e.g., application of a specific chemical inducer, or by hybridization with a second plant line, providing the possibility of inducing thioredoxin transcription only when desired.

2. Transcriptional Terminators

A variety of transcriptional terminators are available for use in expression cassettes. These are responsible for the termination of transcription beyond the transgene and its correct polyadenylation. Appropriate transcriptional terminators and those which are known to function in plants and include the CaMV 35S terminator, the *tml* terminator, the nopaline synthase terminator, the pea *rbcS* E9 terminator. These can be used in both monocotyledons and dicotyledons.

3. Sequences for the Enhancement or Regulation of Expression

Numerous sequences have been found to enhance gene expression from within the transcriptional unit and these sequences can be used in conjunction with the genes of this invention to increase their expression in transgenic plants.

Various intron sequences have been shown to enhance expression, particularly in monocotyledonous cells. For example, the introns of the maize *Adh1* gene have been found to significantly enhance the expression of the wild-type gene under its cognate promoter when introduced into maize cells. Intron 1 was found to be particularly effective and enhanced expression in fusion constructs with the chloramphenicol acetyltransferase gene (Callis *et al.*, Genes Develop 1: 1183-1200 (1987)). In the same experimental system, the intron from the maize *bronze1* gene had a similar effect in enhancing expression. Intron sequences have been routinely incorporated into plant transformation vectors, typically within the non-translated leader.

A number of non-translated leader sequences derived from viruses are also known to enhance expression, and these are particularly effective in dicotyledonous cells. Specifically, leader sequences from Tobacco Mosaic Virus (TMV, the "Ω-sequence"), Maize Chlorotic Mottle Virus (MCMV), and Alfalfa Mosaic Virus (AMV) have been shown to be

effective in enhancing expression (*e.g.* Gallie *et al.* Nucl. Acids Res. 15: 8693-8711 (1987); Skuzeski *et al.* Plant Molec. Biol. 15; 65-79 (1990)).

4. Targeting of the Gene Product Within the Cell

Various mechanisms for targeting gene products are known to exist in plants and the sequences controlling the functioning of these mechanisms have been characterized in some detail. Aminoterminal sequences are responsible for targeting to the ER, the apoplast, and extracellular secretion from aleurone cells (Koehler & Ho, Plant Cell 2: 769-783 (1990)). Additionally, aminoterminal sequences in conjunction with carboxyterminal sequences are responsible for vacuolar targeting of gene products (Shinshi *et al.* Plant Molec. Biol. 14: 357-368 (1990)). By the fusion of the appropriate targeting sequences described above to transgene sequences of interest it is possible to direct the transgene product to any organelle or cell compartment. The signal sequence selected should include the known cleavage site and the fusion constructed should take into account any amino acids after the cleavage site which are required for cleavage. In some cases this requirement may be fulfilled by the addition of a small number of amino acids between the cleavage site and the transgene ATG or alternatively replacement of some amino acids within the transgene sequence.

The above-described mechanisms for cellular targeting can be utilized not only in conjunction with their cognate promoters, but also in conjunction with heterologous promoters so as to effect a specific cell targeting goal under the transcriptional regulation of a promoter which has an expression pattern different to that of the promoter from which the targeting signal derives.

Examples of Expression Cassette Construction

The present invention encompasses the expression of thioredoxin genes under the regulation of any promoter that is expressible in plants, regardless of the origin of the promoter.

Furthermore, the invention encompasses the use of any plant-expressible promoter in conjunction with any further sequences required or selected for the expression of the thioredoxin or thioredoxin reductase gene. Such sequences include, but are not restricted to, transcriptional terminators, extraneous sequences to enhance expression (such as introns [*e.g. Adh* intron 1], viral sequences [*e. g.* TMV-Ω]), and sequences intended for the targeting of the gene product to specific organelles and cell compartments.

Various chemical regulators may be employed to induce expression of the thioredoxin or thioredoxin reductase coding sequence in the plants transformed according to the present invention. In the context of the instant disclosure, "chemical regulators" include chemicals known to be inducers for the PR-1a promoter in plants (described in US 5,614,395), or close derivatives thereof. A preferred group of regulators for the chemically inducible genes of this invention is based on the benzo-1,2,3-thiadiazole (BTH) structure and includes, but is not limited to, the following types of compounds: benzo-1,2,3-thiadiazolecarboxylic acid, benzo-1,2,3-thiadiazolethiocarboxylic acid, cyanobenzo-1,2,3-thiadiazole, benzo-1,2,3-thiadiazolecarboxylic acid amide, benzo-1,2,3-thiadiazolecarboxylic acid hydrazide, benzo-1,2,3-thiadiazole-7-carboxylic acid, benzo-1,2,3-thiadiazole-7-thiocarboxylic acid, 7-cyano-benzo-1,2,3-thiadiazole, benzo-1,2,3-thiadiazole-7-carboxylic acid amide, benzo-1,2,3-thiadiazole-7-carboxylic acid hydrazide, alkyl benzo-1,2,3-thiadiazolecarboxylate in which the alkyl group contains one to six carbon atoms, methyl benzo-1,2,3-thiadiazole-7-carboxylate, n-propyl benzo-1,2,3-thiadiazole-7-carboxylate, benzyl benzo-1,2,3-thiadiazole-7-carboxylate, benzo-1,2,3-thiadiazole-7-carboxylic acid sec-butylhydrazide, and suitable derivatives thereof. Other chemical inducers may include, for example, benzoic acid, salicylic acid (SA), polyacrylic acid and substituted derivatives thereof; suitable substituents include lower alkyl, lower alkoxy, lower alkylthio, and halogen. Still another group of regulators for the chemically inducible DNA sequences of this invention is based on the pyridine carboxylic acid structure, such as the isonicotinic acid structure and preferably the haloisonicotinic acid structure. Preferred are dichloroisonicotinic acids and derivatives thereof, for example the lower alkyl esters. Suitable regulators of this class of compounds are, for example, 2,6-dichloroisonicotinic acid (INA), and the lower alkyl esters thereof, especially the methyl ester.

Constitutive Expression: the Actin Promoter

Several isoforms of actin are known to be expressed in most cell types and consequently the actin promoter is a good choice for a constitutive promoter. In particular, the promoter from the rice *Act1* gene has been cloned and characterized (McElroy *et al.* Plant Cell 2: 163-171 (1990)). A 1.3 kb fragment of the promoter was found to contain all the regulatory elements required for expression in rice protoplasts. Furthermore, numerous expression vectors based on the *Act1* promoter have been constructed specifically for use in monocotyledons (McElroy *et al.* Mol. Gen. Genet. 231: 150-160 (1991)). These incorporate the *Act1*-intron 1, *Adh1* 5' flanking sequence and *Adh1*-intron 1 (from the maize alcohol dehydrogenase gene) and sequence from the CaMV 35S promoter. Vectors showing

highest expression were fusions of 35S and the *Act1* intron or the *Act1* 5' flanking sequence and the *Act1* intron. Optimization of sequences around the initiating ATG (of the GUS reporter gene) also enhanced expression. The promoter expression cassettes described by McElroy *et al.* (Mol. Gen. Genet. 231: 150-160 (1991)) can be easily modified for the expression of genes of the invention and are particularly suitable for use in monocotyledonous hosts. For example, promoter containing fragments can be removed from the McElroy constructions and used to replace the double 35S promoter in pCGN1761ENX, which is then available for the insertion or specific gene sequences. The fusion genes thus constructed can then be transferred to appropriate transformation vectors. In a separate report the rice *Act1* promoter with its first intron has also been found to direct high expression in cultured barley cells (Chibbar *et al.* Plant Cell Rep. 12: 506-509 (1993)).

Constitutive Expression: the Ubiquitin Promoter

Ubiquitin is another gene product known to accumulate in many cell types and its promoter has been cloned from several species for use in transgenic plants (*e.g.* sunflower - Binet *et al.* Plant Science 79: 87-94 (1991), maize - Christensen *et al.* Plant Molec. Biol. 12: 619-632 (1989)). The maize ubiquitin promoter has been developed in transgenic monocot systems and its sequence and vectors constructed for monocot transformation are disclosed in the patent publication EP 0 342 926. Further, Taylor *et al.* (Plant Cell Rep. 12: 491-495 (1993)) describe a vector (pAHC25) which comprises the maize ubiquitin promoter and first intron and its high activity in cell suspensions of numerous monocotyledons when introduced via microprojectile bombardment. The ubiquitin promoter is suitable for the expression of thioredoxin genes in transgenic plants, especially monocotyledons. Suitable vectors are derivatives of pAHC25 or any of the transformation vectors described in this application, modified by the introduction of the appropriate ubiquitin promoter and/or intron sequences.

Root Specific Expression

Another desirable pattern of expression for the thioredoxins and thioredoxin reductases of the instant invention is root expression, for example to enhance extraction of starches and sugars from root crops such as sugarbeets and potatoes. A suitable root promoter is that described by de Framond (FEBS 290: 103-106 (1991)) and also in the published patent application EP 0 452 269. This promoter is transferred to a suitable vector such as pCGN1761ENX for the insertion of a thioredoxin or thioredoxin reductase gene and

subsequent transfer of the entire promoter-gene-terminator cassette to a transformation vector of interest.

Wound Inducible Promoters

Wound-inducible promoters may also be suitable for the expression of thioredoxin genes, which are activated upon harvest. Numerous such promoters have been described (*e.g.* Xu *et al.* Plant Molec. Biol. 22: 573-588 (1993), Logemann *et al.* Plant Cell 1: 151-158 (1989), Rohrmeier & Lehle, Plant Molec. Biol. 22: 783-792 (1993), Firek *et al.* Plant Molec. Biol. 22: 129-142 (1993), Warner *et al.* Plant J. 3: 191-201 (1993)) and all are suitable for use with the instant invention. Logemann *et al.* describe the 5' upstream sequences of the dicotyledonous potato *wun1* gene. Xu *et al.* show that a wound inducible promoter from the dicotyledon potato (*pin2*) is active in the monocotyledon rice. Further, Rohrmeier & Lehle describe the cloning of the maize *Wip1* cDNA which is wound induced and which can be used to isolated the cognate promoter using standard techniques. Similarly, Firek *et al.* and Warner *et al.* have described a wound induced gene from the monocotyledon *Asparagus officinalis* which is expressed at local wound and pathogen invasion sites. Using cloning techniques well known in the art, these promoters can be transferred to suitable vectors, fused to the thioredoxin genes of this invention, and used to express these genes at the sites of plant wounding.

Expression with Plastid Targeting

Chen & Jagendorf (J. Biol. Chem. 268: 2363-2367 (1993) have described the successful use of a chloroplast transit peptide for import of a heterologous transgene. This peptide used is the transit peptide from the *rbcS* gene from *Nicotiana plumbaginifolia* (Poulsen *et al.* Mol. Gen. Genet. 205: 193-200 (1986)). Using the restriction enzymes *DraI* and *SphI*, or *Tsp509I* and *SphI* the DNA sequence encoding this transit peptide can be excised from plasmid prbcS-8B and manipulated for use with any of the constructions described above. The *DraI-SphI* fragment extends from -58 relative to the initiating *rbcS* ATG to, and including, the first amino acid (also a methionine) of the mature peptide immediately after the import cleavage site, whereas the *Tsp509I-SphI* fragment extends from -8 relative to the initiating *rbcS* ATG to, and including, the first amino acid of the mature peptide. Thus, these fragments can be appropriately inserted into the polylinker of any chosen expression cassette generating a transcriptional fusion to the untranslated leader of the chosen promoter (*e.g.* 35S, PR-1a, actin, ubiquitin *etc.*), whilst enabling the insertion of a

thioredoxin or thioredoxin reductase gene in correct fusion downstream of the transit peptide.

Transformation of Dicotyledons

Transformation techniques for dicotyledons are well known in the art and include *Agrobacterium*-based techniques and techniques which do not require *Agrobacterium*. Non-*Agrobacterium* techniques involve the uptake of exogenous genetic material directly by protoplasts or cells. This can be accomplished by PEG or electroporation mediated uptake, particle bombardment-mediated delivery, or microinjection. Examples of these techniques are described by Paszkowski *et al.*, EMBO J 3: 2717-2722 (1984), Potrykus *et al.*, Mol. Gen. Genet. 199: 169-177 (1985), Reich *et al.*, Biotechnology 4: 1001-1004 (1986), and Klein *et al.*, Nature 327: 70-73 (1987). In each case the transformed cells are regenerated to whole plants using standard techniques known in the art.

Agrobacterium-mediated transformation is a preferred technique for transformation of dicotyledons because of its high efficiency of transformation and its broad utility with many different species. The many crop species which are routinely transformable by *Agrobacterium* include tobacco, tomato, sunflower, cotton, oilseed rape, potato, soybean, alfalfa and poplar see, e.g. EP 0 317 511 (cotton), EP 0 249 432 (tomato), WO 87/07299 (*Brassica*), or US 4,795,855 (poplar). *Agrobacterium* transformation typically involves the transfer of the binary vector carrying the foreign DNA of interest (*e.g.* pCIB200 or pCIB2001) to an appropriate *Agrobacterium* strain which may depend of the complement of *vir* genes carried by the host *Agrobacterium* strain either on a co-resident Ti plasmid or chromosomally (*e.g.* strain CIB542 for pCIB200 and pCIB2001 (Uknes *et al.* Plant Cell 5: 159-169 (1993)). The transfer of the recombinant binary vector to *Agrobacterium* is accomplished by a triparental mating procedure using *E. coli* carrying the recombinant binary vector, a helper *E. coli* strain which carries a plasmid such as pRK2013 and which is able to mobilize the recombinant binary vector to the target *Agrobacterium* strain. Alternatively, the recombinant binary vector can be transferred to *Agrobacterium* by DNA transformation (Höfgen & Willmitzer, Nucl. Acids Res. 16: 9877(1988)).

Transformation of the target plant species by recombinant *Agrobacterium* usually involves co-cultivation of the *Agrobacterium* with explants from the plant and follows protocols well known in the art. Transformed tissue is regenerated on selectable medium carrying the

antibiotic or herbicide resistance marker present between the binary plasmid T-DNA borders.

Transformation of dicots may also be carried out using biolistics. Particularly preferred for soybean transformation is the method described in US 5024944.

Transformation of Monocotyledons

Transformation of most monocotyledon species has now also become routine. Preferred techniques include direct gene transfer into protoplasts using PEG or electroporation techniques, and particle bombardment into callus tissue. Transformations can be undertaken with a single DNA species or multiple DNA species (*i.e.* co-transformation) and both these techniques are suitable for use with this invention. Co-transformation may have the advantage of avoiding complex vector construction and of generating transgenic plants with unlinked loci for the gene of interest and the selectable marker, enabling the removal of the selectable marker in subsequent generations, should this be regarded desirable. However, a disadvantage of the use of co-transformation is the less than 100% frequency with which separate DNA species are integrated into the genome (Schocher *et al.* Biotechnology 4: 1093-1096 (1986)).

Patent applications EP 0 292 435, EP 0 392 225 and WO 93/07278 describe techniques for the preparation of callus and protoplasts from an élite inbred line of maize, transformation of protoplasts using PEG or electroporation, and the regeneration of maize plants from transformed protoplasts. Gordon-Kamm *et al.* (Plant Cell 2: 603-618 (1990)) and Fromm *et al.* (Biotechnology 8: 833-839 (1990)) have published techniques for transformation of A188-derived maize line using particle bombardment. Furthermore, international patent application WO 93/07278 and Koziel *et al.* (Biotechnology 11: 194-200 (1993)) describe techniques for the transformation of élite inbred lines of maize by particle bombardment. This technique utilizes immature maize embryos of 1.5-2.5 mm length excised from a maize ear 14-15 days after pollination and a PDS-1000He Biolistics device for bombardment.

Maize may also be transformed by Agrobacterium, e.g., using the methods described in Ishida et al., 1996; High efficiency transformation of maize (Zea mays L.) mediated by *Agrobacterium* tumefaciens, Nature Biotechnology 14, 745-750.

Transformation of rice can also be undertaken by direct gene transfer techniques utilizing protoplasts or particle bombardment. Protoplast-mediated transformation has been described for *Japonica*-types and *Indica*-types (Zhang *et al.*, Plant Cell Rep 7: 379-384

(1988); Shimamoto *et al.* Nature 338: 274-277 (1989); Datta *et al.* Biotechnology 8: 736-740 (1990)). Both types are also routinely transformable using particle bombardment (Christou *et al.* Biotechnology 9: 957-962 (1991)).

Patent application EP 0 332 581 describes techniques for the generation, transformation and regeneration of Pooideae protoplasts. These techniques allow the transformation of *Dactylis* and wheat. Furthermore, wheat transformation was been described by Vasil *et al.* (Biotechnology 10: 667-674 (1992)) using particle bombardment into cells of type C long-term regenerable callus, and also by Vasil *et al.* (Biotechnology 11: 1553-1558 (1993)) and Weeks *et al.* (Plant Physiol. 102: 1077-1084 (1993)) using particle bombardment of immature embryos and immature embryo-derived callus. A preferred technique for wheat transformation, however, involves the transformation of wheat by particle bombardment of immature embryos and includes either a high sucrose or a high maltose step prior to gene delivery. Prior to bombardment, any number of embryos (0.75-1 mm in length) are plated onto MS medium with 3% sucrose (Murashige & Skoog, Physiology Planetarium 15: 473-497 (1962)) and 3 mg/l 2,4-D for induction of somatic embryos which is allowed to proceed in the dark. On the chosen day of bombardment, embryos are removed from the induction medium and placed onto the osmoticum (*i.e.* induction medium with sucrose or maltose added at the desired concentration, typically 15%). The embryos are allowed to plasmolyze for 2-3 h and are then bombarded. Twenty embryos per target plate is typical, although not critical. An appropriate gene-carrying plasmid (such as pCIB3064 or pSG35) is precipitated onto micrometer size gold particles using standard procedures. Each plate of embryos is shot with the DuPont Biolistics® helium device using a burst pressure of ~1000 psi using a standard 80 mesh screen. After bombardment, the embryos are placed back into the dark to recover for about 24 h (still on osmoticum). After 24 hrs, the embryos are removed from the osmoticum and placed back onto induction medium where they stay for about a month before regeneration. Approximately one month later the embryo explants with developing embryogenic callus are transferred to regeneration medium (MS + 1 mg/liter NAA, 5 mg/liter GA), further containing the appropriate selection agent (10 mg/l Basta in the case of pCIB3064 and 2 mg/l methotrexate in the case of pSOG35). After approximately one month, developed shoots are transferred to larger sterile containers known as "GA7s" which contained half-strength MS, 2% sucrose, and the same concentration of selection agent. Patent application WO 94/13822 describes methods for wheat transformation and is hereby incorporated by reference.

Plastid Transformation

Plastid transformation technology is extensively described in U.S. Patent Nos. 5,451,513, 5,545,817, and 5,545,818, all of which are hereby expressly incorporated by reference in their entireties; in PCT application nos. WO 95/16783 and WO 98/11235, which are hereby incorporated by reference in its entirety; and in McBride *et al.* (1994) Proc. Natl. Acad. Scl. USA 91, 7301-7305, which is also hereby incorporated by reference in its entirety; The basic technique for chloroplast transformation involves introducing regions of cloned plastid DNA flanking a selectable marker together with the gene of interest into a suitable target tissue, e.g., using biolistics or protoplast transformation (e.g., calcium chloride or PEG mediated transformation). The 1 to 1.5 kb flanking regions, termed targeting sequences, facilitate homologous recombination with the plastid genome and thus allow the replacement or modification of specific regions of the plastome. Initially, point mutations in the chloroplast 16S rRNA and rps12 genes conferring resistance to spectinomycin and/or streptomycin were utilized as selectable markers for transformation (Svab, Z., Hajdukiewicz, P., and Maliga, P. (1990) Proc. Natl. Acad. Sci. USA 87, 8526-8530, hereby incorporated by reference; Staub, J. M., and Maliga, P. (1992) Plant Cell 4, 39-45, hereby incorporated by reference). This resulted in stable homoplasmic transformants at a frequency of approximately one per 100 bombardments of target leaves. The presence of cloning sites between these markers allowed creation of a plastid targeting vector for introduction of foreign genes (Staub, J.M., and Maliga, P. (1993) *EMBO J.* 12, 601-606, hereby incorporated by reference). Substantial increases in transformation frequency were obtained by replacement of the recessive rRNA or r-protein antibiotic resistance genes with a dominant selectable marker, the bacterial *aadA* gene encoding the spectinomycin-detoxifying enzyme aminoglycoside-3'-adenyltransferase (Svab, Z., and Maliga, P. (1993) *Proc. Natl. Acad. Sci. USA* 90, 913-917, hereby incorporated by reference). Previously, this marker had been used successfully for high-frequency transformation of the plastid genome of the green alga *Chlamydomonas reinhardtii* (Goldschmidt-Clermont, M. (1991) *Nucl. Acids Res.* 19, 4083-4089, hereby incorporated by reference). Other selectable markers useful for plastid transformation are known in the art and encompassed within the scope of the invention. Typically, approximately 15-20 cell division cycles following transformation are required to reach a homoplastidic state.

Plastid expression, in which genes are inserted by homologous recombination into all of the several thousand copies of the circular plastid genome present in each plant cell, takes

advantage of the enormous copy number advantage over nuclear-expressed genes to permit expression levels that can readily exceed 10% of the total soluble plant protein. However, such high expression levels may pose potential viability problems, especially during early plant growth and development. Similar problems are posed by the expression of bioactive enzymes or proteins that may be highly deleterious to the survival of transgenic plants and hence if expressed constitutively may not be introduced successfully into the plant genome. Thus, in one aspect, the present invention has coupled expression in the nuclear genome of a chloroplast-targeted phage T7 RNA polymerase under control of the chemically inducible PR-1a promoter (US 5,614,395 incorporated by reference) of tobacco to a chloroplast reporter transgene regulated by T7 gene 10 promoter/terminator sequences. For example, when plastid transformants homoplasmic for the maternally inherited *uidA* gene encoding the β-glucuronidase (GUS) reporter are pollinated by lines expressing the T7 polymerase in the nucleus, F1 plants are obtained that carry both transgene constructs but do not express the GUS protein. Synthesis of large amounts of enzymatically active GUS is triggered in plastids of these plants only after foliar application of the PR-1a inducer compound benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH).

BRIEF DESCRIPTION OF THE SEQUENCES IN THE SEQUENCE LISTING

SEQ ID NO:1 protein sequence of thioredoxin from *Methanococcus jannaschii*

SEQ ID NO:2 protein sequence of thioredoxin from *Archaeoglobus fulgidus* (trx-1)

SEQ ID NO:3 protein sequence of thioredoxin from *Archaeoglobus fulgidus* (trx-2)

SEQ ID NO:4 protein sequence of thioredoxin from *Archaeoglobus fulgidus* (trx-3)

SEQ ID NO:5 protein sequence of thioredoxin from *Archaeoglobus fulgidus* (trx-4)

SEQ ID NO:6 protein sequence of thioredoxin reductase from *Methanococcus jannaschii* (trxB)

SEQ ID NO:7 protein sequence of thioredoxin reductase from *Archaeoglobus fulgidus* (trxB)

SEQ ID NO:8 Clontech sequence

SEQ ID NO:9 Joshi sequence

EXAMPLES

The invention is further described by reference to the following detailed examples. These

examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified.

Standard recombinant DNA and molecular cloning techniques used here are well known in the art and are described, for example, by Sambrook et al. (1989) Molecular Cloning and by Ausubel et al. (1994) Current Protocols in Molecular Biology.

Example 1: Transformation of maize with heat-stable thioredoxin

A gene expressing the heat-stable thioredoxin from Methanococcus jannaschii, having the sequence

MSKVKIELFTSPMCPHCPAAKRVVEEVANEMPDAVEVEYINVMENPQKAMEYGIMAVPTIVI NGDVEFIGAPTKEALVEAIKKRL (SEQ ID NO:1)

is prepared using maize preferred codons as described in US patent 5625136, under control of the seed-specific gamma-zein promoter, and the expression cassette incorporated between the T-DNA boundaries of the pGIGUP plasmid. The T-DNA of this plasmid contains a plant expressible bar gene driven by the ubiquitin promoter (Christensen et al., Plant Mol. biol. 18: 875-689, 1992) to provide resistance to phosphinothricin. It also contains the GUS gene (beta-glucuronidase) with an intron in the N-terminal codon of the coding sequence driven by a chimeric promoter (SMAS) derived from the octopine and mannopine synthase genes (a trimer of the octopine synthase promoter upstream activating sequence with a domain of the mannopine synthase gene, Ni et al., Plant J. 7: 661-676, 1995). This intron-GUS gene expresses GUS activity in plant cells but not in *Agrobacterium*. Alternatively, the heat-stable thioredoxin from *Methanococcus jannaschii* is cloned into the plasmid pNOV117 which contains a plant expressible-*pmi* gene driven by the maize ubiquitin promoter for selection on mannose (Christensen *et al.*, 1992, Joersbo *et al.*, 1998).

Strain *A. tumefaciens* LBA4404 (pAL4404, pSB1) is used in these experiments. pAL4404 is a disarmed helper plasmid. pSB1 is a wide host range plasmid that contains a region of homology to pGIGUP and pNOV117 and a 15.2 kb KpnI fragment from the virulence region of pTiBo542 (Ishida et al., 1996; High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*, Nature Biotechnology 14, 745-750). The introduction of the plasmid pGIGUP or pNOV117 by electroporation into LBA4404 (pAL4404, pSB1) results in a cointegration of pGIGUP or pNOV117 and pSB1. The T-DNA of pNOV117 contains a mannose-6-phosphate isomerase gene driven by the ubiquitin

promoter to provide the ability to metabolize mannose, as well as the thioredoxin gene described above.

Agrobacterium is grown for 3 days on YP medium (5g/l yeast extract, 10g/l peptone, 5g/l NaCl, 15 g/l agar, pH 6.8) supplemented with 50 mg/l spectinomycin and 10 mg/l tetracycline. Bacteria are collected with a loop and suspended in N6 liquid medium at a density ranging from 10^9 to 5 10^9 cells/ml. *Agrobacterium* cells can also be collected from an overnight culture in YP medium and resuspended in N6 liquid medium. For 1L of medium add: 4g powdered N6 salts (Sigma, St. Louis, MO), 30g sucrose, 100 mg myo-inositol, 2 mg glycine, 1 mg thiamine, 0.5 mg pyrodoxine HCL, 0.5 mg nicotinic acid, 2 mg 2,4-D (from stock solution [1 mg/mL] made by dissolving 2,4-D in dilute KOH). Adjust to pH 6.0 with 1M KOH, add 3 g Gelrite, and autoclave.

Maize immature embryos are obtained approximately 10 to 14 days after self-pollination. The immature zygotic embryos are divided among different plates containing medium capable of inducing and supporting embryogenic callus formation at about 25 immature embryos per plate.

The immature embryos are inoculated either on the plate or in liquid with *Agrobacterium* having a Ti plasmid comprising a selectable marker gene. The immature embryos are plated on callus initiation medium containing silver nitrate (10 mg/l) either prior or immediately after inoculation with *Agrobacterium*. Approximately 25 immature embryos are placed onto each plate. 16 to 72 hours after inoculation, immature embryos are transferred to callus initiation medium with silver nitrate and cefotaxim. Selection of transformed cells is carried out as follows:

Mannose is used to select transformed cells *in vitro*. This selection can be applied as low as 1 g/L 2 to 20 days after inoculation and maintained for a total of 2-12 weeks. The embryogenic callus so obtained is regenerated in the presence or absence of mannose on standard medium of regeneration. All plants are tested by the chlorophenol red (CR) test for tolerance to mannose. This assay utilizes a pH sensitive indicator dye to show which cells are growing in the presence of mannose. Cells that grow produce a pH change in the media and turn the indicator Chlorophenol Red (CR) yellow from red. Plants expressing the tolerance to mannose are easily identified in this test. Plants positive by the CR test are assayed by PCR for the presence of the mannose gene. Plants which are positive for PCR test are analyzed by Southern blot.

The regenerated plants are assayed for expression of the thioredoxin. The plants are developmentally normal. Corn grain from progeny plants derived from the highest

expressing event is assayed in a small scale wet milling process and starch extractability is measured compared to corn of the same genotype without the thioredoxin transgene. Corn expressing the thioredoxin gene exhibits substantially greater starch availability in the wet-milling process than the isogenic non-transformed corn.

Example 2: Transformation of maize with heat-stable thioredoxin and thioredoxin reductase

Using the procedures described in Example 1, maize is co-transformed with genes for both thioredoxin and thioredoxin reductase from *M. jannaschii*, described above. Both genes are under control of the seed specific gamma zein promoter. The two genes are linked and placed between the right and left borders of the pGIGUP or pNOV117 plasmid to enhance the likelihood that both genes will be incorporated into the chromosome of the plant as a single insert.

The regenerated plants are assayed for expression of the thioredoxin and thioredoxin reductase. The plants are developmentally normal. Corn grain from progeny plants derived from the highest expressing event is assayed in a small scale wet milling process and starch extractability is measured compared to corn of the same genotype without the thioredoxin/thioredoxin reductase transgenes. Corn expressing the thioredoxin and thioredoxin reductase genes exhibits substantially greater starch availability in the wet-milling process than the isogenic non-transformed corn.

What is claimed is:

1. A method to increase efficiency of separation of starch and protein in a grain milling process, comprising steeping the grain at an elevated temperature in the presence of supplemental thioredoxin and/or thioredoxin reductase and separating the starch and protein components of the grain.

2. The method of claim 1 wherein the grain includes grain from a transgenic plant wherein the transgene expresses thioredoxin and/or thioredoxin reductase.

3. The method of claim 2 wherein the plant is selected from corn (*Zea mays*) and soybean.

4. A plant comprising a heterologous DNA sequence coding for a thioredoxin and/or thioredoxin reductase stably integrated into its nuclear or plastid DNA.

5. The plant according to claim 4, wherein the thioredoxin and/or thioredoxin reductase is thermostable.

6. The plant of claim 5 wherein the thioredoxin and/or thioredoxin reductase is selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, and SEQ ID NO: 7.

7. The plant of any one of claims 4 to 6 wherein the plant is selected from corn and soybean.

8. A plant expressible expression cassette comprising a coding region for a thioredoxin and/or thioredoxin reductase operably linked to promoter and terminator sequences which function in a plant.

9. The plant expressible expression cassette according to claim 8, wherein the thioredoxin and/or thioredoxin reductase is thermostable.

10. The plant expressible expression cassette of claim 9, wherein the thioredoxin and/or thioredoxin reductase is selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, and SEQ ID NO: 7.

11. A method of producing grain comprising high levels of thioredoxin and/or thioredoxin reductase comprising transforming plants with an expression cassette of claims 8 to 10.

12. A method of producing grain comprising high levels of thioredoxin and/or thioredoxin reductase comprising
pollinating a first plant comprising a heterologous expression cassette comprising a transactivator-regulated promoter regulated and operably linked to a DNA sequence coding for a thioredoxin and/or thioredoxin reductase, with pollen from a second plant comprising a heterologous expression cassette comprising a promoter operably linked to a DNA sequence coding for a transactivator capable of regulating said transactivator-regulated promoter; and
recovering grain from the plant thus pollinated.

13. Use of plants or plant material according to any one of claims 4 to 7 as animal feed.

SEQUENCE LISTING

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INTERNATIONAL SEARCH REPORT

Inte. .onal Application No
PCT/EP 99/09986

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C12N15/53 C12N9/02 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C08B C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LEE H B ET AL: "INCORPORATION OF FOREIGN GENE WITH TI PLASMID VECTOR SYSTEM II. EXPRESSIO OF ESCHERICHIA-COLI THIOREDOXIN GENE IN CULTURED TOBACCO CELLS." KOREAN BIOCHEM J, (1988) 21 (4), 384-388, XP000885808 the whole document ---	4,5,7-9, 11-13
X	SHI JINRUI ET AL: "A novel plasma membrane-bound thioredoxin from soybean." PLANT MOLECULAR BIOLOGY 1996, vol. 32, no. 4, 1996, pages 653-662, XP000885857 ISSN: 0167-4412 the whole document ---	4,5,7-9, 11-13

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 April 2000	02/05/2000
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Morawetz, R

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/EP 99/09986

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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